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Construction, characterization and preclinical evaluation of MTBVAC, the first live-attenuated *M. tuberculosis*-based vaccine to enter clinical trials

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ABSTRACT

The development of a new tuberculosis vaccine is an urgent need due to the failure of the current vaccine, BCG, to protect against the respiratory form of the disease. MTBVAC is an attenuated *Mycobacterium tuberculosis* vaccine candidate genetically engineered to fulfil the Geneva consensus requirements to enter human clinical trials. We selected a *M. tuberculosis* clinical isolate to generate two independent deletions without antibiotic-resistance markers in the genes *phoP*, coding for a transcription factor key for the regulation of *M. tuberculosis* virulence, and *fadD26*, essential for the synthesis of the complex lipids phthiocerol dimycocerosates (DIM), one of the major mycobacterial virulence factors. The resultant strain MTBVAC exhibits safety and biodistribution profiles similar to BCG and confers superior protection in preclinical studies. These features have enabled MTBVAC to be the first live attenuated *M. tuberculosis* vaccine to enter clinical evaluation.

1. Introduction

The only vaccine against tuberculosis (TB) in use today, Bacille Calmette-Guerin (BCG), is a live attenuated strain of *Mycobacterium bovis* effective in reducing the rate of severe forms of TB (meningitis and miliary TB) in children, but is inconsistent in preventing spread of pulmonary TB, the most common form of the disease in adolescents and adults, which fuels the continuing epidemic [1, 2]. Developed a century ago by repeated subculture, the principal genetic basis for BCG attenuation is the loss of RD1 region, which encodes the machinery required to synthesize and export the major T-cell antigen/virulence factor ESAT-6/CFP-10 [3, 4]. Subsequent worldwide distribution of BCG and repeated subculture in non-standardized conditions has led to emergence of a number of daughter BCG sub-strains, which comprise natural mutants of well-recognized virulence factors, suggesting that some sub-strains may be more attenuated and otherwise less immunogenic than others [3, 5-7]. New vaccines able to prevent respiratory forms of TB will have a tremendous impact in preventing transmission and control of the disease [8-10].

We have previously described the construction of the SO2 strain by insertion of a kanamycin-resistance cassette in the *phoP* gene of a *M. tuberculosis* clinical isolate [11]. PhoP is a key transcriptional regulator, which controls approximately 2% of *M. tuberculosis* coding capacity, including the synthesis of the immunomodulatory trehalose-derived lipids, diacyl- (DAT) and polyacyl-trehaloses (PAT), and the secretion of the virulence factor ESAT-6 [12-15]. Ten years of rigorous preclinical testing of SO2 as a vaccine candidate has provided robust data for its high degree of safety and improved immunogenicity and protective efficacy compared to BCG in relevant animal models of TB, from mice to non-human primates [16-19]. Despite the promising results, the establishment of the Geneva consensus for new live mycobacterial vaccines, demanding the presence of two stable independent mutations without antibiotic-resistance markers for *M. tuberculosis*-based candidates, in addition to a safety and efficacy

74 profile at least comparable to BCG in the relevant animal models, rendered SO2 unsuitable
75 for entry into clinical trials [20, 21].
76
77 Here we describe the construction and extensive preclinical characterization of MTBVAC, the
78 first live-attenuated vaccine based on a *M. tuberculosis* human isolate that entered first-in-
79 human clinical evaluation in January 2013. This vaccine is based on the prototype SO2 and
80 is genetically engineered to fulfil the Geneva consensus requirements for progressing new
81 live mycobacterial vaccines into Phase 1 clinical trials, requiring two non-reverting unmarked
82 independent mutations [20]. MTBVAC contains two stable deletions in the *phoP* and *fadD26*
83 genes without antibiotic-resistance markers. FadD26 is essential for the synthesis of
84 phthiocerol dimycocerosates (DIM), a family of surface lipids involved in *M. tuberculosis*
85 virulence [22, 23]. MTBVAC is safe in all preclinical studies and confers superior protection in
86 mice compared to the reference licensed strain BCG Danish 1331 used in the clinic.

2. Materials and Methods

2.1. MTBVAC construction and culture conditions

M. tuberculosis Mt103, the parental strain in this study, was isolated from an immunocompentent TB patient [24]. MTBVAC vaccine candidate was constructed following standard mycobacterial genetic-engineering protocols [25-27] (for a detailed description of the process see supplementary methods).

Mycobacterial strains were grown at 37°C in Middlebrook 7H9 broth (Difco) supplemented with albumin, dextrose and catalase (ADC) (Difco) and 0.05% (v/v) Tween-80, or on solid Middlebrook 7H10 or 7H11 (Difco) supplemented with ADC (Difco). When required, kanamycin (20 μg/ml) was added to the media. All chemicals were purchased from Sigma-Aldrich except where indicated.

2.2. *Protein isolation and western-blot analysis*

Whole-cell proteins. Mycobacteria from early log-phase liquid cultures of Mt103 or MTBVAC were pelleted by centrifugation, washed and resuspended in phosphate-buffered saline (PBS) containing a cocktail of protease inhibitors. Cell suspensions were disrupted by sonication.

Secreted proteins. M. tuberculosis strains were grown in 7H9 supplemented with dextrose (2 g/l) and supernatants were separated by centrifugation. Secreted proteins were precipitated by incubation with 10% (v/v) trichloroacetic acid for 30 min on ice and then centrifuged at 4ºC for 30 min. Pelleted proteins were rinsed with cold acetone and resuspended in 150 mM Tris-HCl pH=8.

Western blot. Both protein preparations were sterilized using a 0.22-μm low protein-binding filter (Pall) and quantified using the RC DC protein assay (BioRad). 5 μg of protein were loaded per well and separated by SDS-PAGE. Immunodetection was carried out using primary monoclonal mouse antibodies anti-ESAT6 or anti-GroEL (Abcam), followed by

incubation with an anti-mouse secondary antibody and developed with a chemiluminiscent substrate (Immobilon™ western, Millipore).

2.3. Complex lipids extraction and thin-layer chromatography analysis

Mt103 and MTBVAC strains were grown in 16 ml of liquid medium for 10 days (exponential phase). Radiolabelling of methyl-branched fatty acids was performed by incubating the cultures with 7 μCi of ^{14}C-propionate (specific activity 55 mCi/mmol; MP Biomedicals) for 24h at 37°C with continuous agitation and mycobacterial lipids were then extracted as previously described [28]. Lipid profiles were analyzed by spotting equivalent amounts of crude extracts (resuspended in $CHCl_3$) on silica gel G60 plates (Merck), which were then run in various solvent systems ($CHCl_3$/CH_3OH/H_2O 60:16:2 (v/v) for DAT; $CHCl_3$/CH_3OH 99:1 (v/v) for PAT; and petroleum ether/diethyl ether 9:1 (v/v) for DIM). Radiolabeled lipids were visualized with a Typhoon PhosphorImager (Amersham Biosciences).

2.4. Animal studies

All the animals were kept under controlled conditions and observed for any sign of disease. Experimental work was conducted in agreement with European and national directives for protection of experimental animals and with approval from the competent local ethical committees.

Mouse studies

For immunogenicity studies, BALB/c mice (Charles River) were mock-treated or subcutaneously inoculated with $5x10^5$ colony forming units (CFU) of reconstituted lyophilized MTBVAC, prototype SO2 or BCG Danish 1331. At 7, 28 and 60 days post-inoculation, splenocytes were collected from 4 animals per group and stimulated overnight with purified protein derivative (PPD) [16]. Intracellular staining of IFNγ was performed using BD Cytofix/Cytoperm Fixation/Permeabilization kit following manufacturer instructions.

For biodistribution studies, groups of male or female BALB/c mice (Charles River) received an intradermal injection of $5x10^5$ CFU of reconstituted lyophilized MTBVAC or BCG Danish 1331. At weeks 1, 2, 4, 8, 16 and 24 post-inoculation, 4 mice per group were randomly selected and sacrificed for enumeration of viable bacteria in inguinal and axillary lymph nodes, spleen, liver, lungs, kidneys, testis, ovaries and brain. Urine and stool samples were collected at each time point. Organs were homogenized and viable bacteria were counted by plating onto selective 7H11-ADC supplemented with antibiotics to avoid contamination.

For protection studies, groups of 8 C57BL/6 mice (Janvier) were mock-treated or subcutaneously vaccinated with $5x10^5$ CFU of reconstituted lyophilized MTBVAC or BCG Danish 1331. 8 weeks post-vaccination, mice were intranasally challenged with 100 CFU of virulent *M. tuberculosis* H37Rv. 4 weeks later, mice were humanely sacrificed and CFU quantified in lungs and spleen.

For safety studies, groups of 12 CB-17/Icr Ico SCID mice (Charles River) received a single subcutaneous administration of $2.5x10^7$ CFU (equivalent to 50 times the dose recommended for BCG in humans, $5x10^5$) of vaccine strains MTBVAC, BCG Pasteur or BCG Danish 1331; a group was inoculated with 10^5 CFU of parental *M. tuberculosis* Mt103 as the virulence control. The endpoint of the experiment was defined as survival up to 13 weeks post-inoculation and then animals were humanely euthanized and bacterial load in lungs and spleen was quantified.

Guinea pig studies

For protection studies, groups of 8 Dunkin-Hartley guinea pigs (Harlan) were mock-treated or subcutaneously vaccinated in the nape of the neck with $5x10^3$, $5x10^4$ and $5x10^5$ CFU of reconstituted lyophilized MTBVAC, or $5x10^4$ CFU of SO2 or BCG Danish 1331. 12 weeks post-vaccination, animals were subjected to an aerosol challenge of 10-50 CFU per lung of *M. tuberculosis* H37Rv (NCTC 7416) [29], using a Henderson apparatus [30]. Bacterial burden in lungs and spleen at 4 weeks post-challenge was quantified. The severity of the

168 microscopic lesions in lungs and spleen was also evaluated by a subjective histopathology
169 scoring matrix [29].
170 For shedding and excretion experiments, groups of 10 Dunkin-Hartley guinea pigs (5 males
171 and 5 females) (Harlan), were intradermally vaccinated with $5x10^5$ CFU of reconstituted
172 lyophilized MTBVAC or BCG Danish 1331. Animals were observed over a period of 7 weeks
173 post-vaccination during which samples (injection site swab, urine and stool) were collected at
174 the selected time points and plated for the detection of possible vaccine shedding or
175 excretion. Injection site swabs were collected at 0, 3, 7, 14, 21, 28, 35, 42 and 49 days post-
176 inoculation. In addition, swabs of the vaccine site were collected immediately on observation
177 of a site 'opening' event. Urine and stool samples were collected at 2, 7, 21 and 49 days
178 post-inoculation.
179 For safety studies, groups of 8 Dunkin-Hartley guinea pigs (Harlan) were subcutaneously
180 vaccinated with $2.5x10^7$ CFU (equivalent to 50 times the dose recommended for BCG in
181 humans, $5x10^5$) of reconstituted lyophilized MTBVAC or BCG Danish 1331. Animals were
182 observed daily over a period of 42 days post-administration and body weights of all animals
183 were recorded weekly. At the end of the experiment, animals were euthanized and examined
184 for any signs of TB lesions in lungs, spleen, liver and lymph nodes. Any potential lesion was
185 recovered and submitted for culture and/or fixed and submitted for histological assessment.

3. Results

3.1. From SO2 to MTBVAC: generation of two unmarked deletions in phoP and fadD26 genes to fulfil the Geneva consensus

Considering that attenuation by two non-reverting independent mutations without antibiotic-resistance markers is required to fulfil the Geneva consensus criteria, we sought to construct an SO2-based vaccine that accomplished these criteria for progressing this vaccine candidate into clinical evaluation [20]. We followed a stepwise approach to genetically engineer two stable deletions in *phoP* and *fadD26* genes in the SO2 strain, with subsequent elimination of antibiotic-resistance markers, generating a novel vaccine candidate that was named MTBVAC (Fig. 1A). No significant differences in growth behaviour were observed between MTBVAC and SO2 in axenic culture (Fig. S3).

To confirm the biochemical phenotype of MTBVAC, the lipid content of the cell wall envelope was analysed by thin-layer chromatography. This analysis confirmed that, due to *fadD26* deletion, the outermost layer of MTBVAC is devoid of DIM [22] (Fig. 1B), and that *phoP* inactivation renders MTBVAC unable to synthesize trehalose-derived lipids DAT and PAT [12] (Fig. 1C). In addition to these characteristic lipid deficiencies, the deletion of *phoP* in MTBVAC reduces the amount of intracellular ESAT-6 and prevents the secretion of this major virulence factor [14] (Fig. 1D).

Once the *in vitro* phenotype provided by the *phoP* and *fadD26* deletions was corroborated, MTBVAC was subjected to an extensive preclinical characterization to support its progress to clinical evaluation.

3.2. MTBVAC has vaccine properties comparable to SO2

To corroborate that the phenotypic equivalence of MTBVAC and SO2 translates to functional comparability, bridging studies for vaccine efficacy in guinea pigs and immunogenicity in mice were conducted.

First, mouse immunogenicity studies, using the clinical dose and route of administration, showed comparable results for MTBVAC and SO2 as measured by the percentage of splenic IFNγ-producing $CD4^+$ cells, following stimulation with *M. tuberculosis* PPD, at different time points post-vaccination (Fig. S4).

Second, in the guinea-pig short-term protection experiment, MTBVAC conferred statistically equivalent protection compared to SO2 both in lungs (Fig. 2A) and spleen (Fig. 2B). Similar protection was provided by all the tested doses of MTBVAC. A comparable protective efficacy was obtained with BCG (data not shown), as previously described for SO2 [16].

3.3. MTBVAC is as safe as the licensed vaccine BCG Danish 1331

To support entry into clinical trials in Europe, a battery of preclinical studies of MTBVAC freeze-dried preparation, produced in compliance with Good Manufacturing Practices (GMP), was conducted in mice and guinea pigs, meeting Regulatory requirements in Spain (country of GMP manufacture) and in Switzerland (country of Phase 1 trial) in accordance with the European Pharmacopoeia monograph [31] and the WHO Recommendations to Assure the Quality, Safety and Efficacy of BCG freeze-dried vaccines for human use [32]. As BCG Danish 1331 is the only licensed TB vaccine in Europe, it was used as the reference comparator in the preclinical characterization of MTBVAC freeze-dried product.

MTBVAC showed a comparable safety profile to BCG Danish 1331 in the survival experiment using immunocompromised SCID mice inoculated with 50 times the recommended human dose for BCG (Table 1). All the SCID mice inoculated with the vaccine strains survived to the end of the experiment. Equivalent bacterial loads both in lungs (Fig. 3A) and spleen (Fig. 3B) were observed for MTBVAC and BCG Danish 1331. In contrast, mice in the Mt103 group died by week six post-inoculation and a significantly higher bacterial burden was observed. In the case of the guinea pig study, none of the animals inoculated with 50 times the BCG dose recommended for humans died or showed signs of TB in-life or at autopsy (table 1).

Vaccine biodistribution in mice was mainly in a localization restricted to lymphoid organs, especially lymph nodes where a peak of colonies was observed between two and four weeks post-vaccination, followed by progressive clearance thereafter (Fig. 4). In addition, MTBVAC and BCG Danish 1331 could not be detected in urine and stool (table 1). In guinea pigs, viable MTBVAC or BCG were uniquely found in the site of vaccination only immediately after administration. In the case of BCG, some “opening” events in the site of vaccination were observed (table 1).

3.4. MTBVAC induces improved protection in mice

Having established the comparable safety and biodistribution profile of MTBVAC and BCG Danish 1331 clinical lots, we conducted a preclinical protection experiment in mice to compare the efficacy of the two vaccines (table 1). Following a two-month vaccination by the clinical route and dose of administration, we compared the efficacy of MTBVAC and BCG Danish 1331 at one month post-challenge with virulent *M. tuberculosis* H37Rv (the reference laboratory strain) by the natural respiratory route of infection (Fig. 5). Even though both vaccines conferred significant protection compared to saline controls, a significantly higher reduction in bacterial burden was observed in MTBVAC group compared to BCG, both in lungs (Fig. 5A) and spleen (Fig 5B).

4. Discussion

One of the main limitations presented with BCG is the large variability in protective efficacy afforded in clinic ranging from 0% to 80% [1, 2]. Thus, a better vaccine that can induce superior protection over BCG and which could last into adolescence and adulthood against pulmonary disease would have a tremendous impact on TB control programs [10]. MTBVAC is the first *M. tuberculosis*-based vaccine candidate to fulfil the Geneva consensus requirements for progressing new live tuberculosis vaccines to clinical trials [20]. Two unmarked deletions have been engineered in the genes *phoP* and *fadD26* to render the final vaccine construct phenotypically comparable to the vaccine prototype SO2 (Fig. 1).

MTBVAC is a derivative of a clinical isolate of *M. tuberculosis*, a classical approach to human vaccinology. Most of the whole cell live vaccines used in human immunization schedules, except for small pox, TB and rotavirus infections, are based on the attenuated pathogen from human origin [33]. MTBVAC is a derivative of a clinical isolate of *M. tuberculosis*, a classical approach to human vaccinology. The scientific rationale behind an *M. tuberculosis*-based vaccine that could replace BCG is that the latter is derived from the bovine pathogen *M. bovis*, an animal adapted close relative of *M. tuberculosis* which lost a series of genes in its genome in the process of its co-evolution with the immune system of its natural host. In addition, when compared to *M. tuberculosis* clinical isolates, more than one hundred genes are absent in BCG genome [3, 34]. These *M. tuberculosis*-restricted genes must be important in the successful interaction with the human immune system. Therefore, a vaccine based on a human pathogen should be more effective at inducing more specific protective immunity against TB in the clinic. To demonstrate this rationale it is imperative to go to human efficacy trials, provided that the current animal models for TB are exhausted. Remarkably, all the current TB vaccine strategies under clinical evaluation are based on BCG [35, 36]. Consequently, the use of a vaccine based on the human pathogen as MTBVAC is a novel strategy.

Results obtained in immunogenicity (Fig. S4) and protective efficacy (Fig. 2) bridging experiments provide evidence that MTBVAC is functionally comparable to its prototype SO2 and, therefore, data generated in preclinical studies with SO2 were accepted by the Swiss Regulatory Authorities as valid to support MTBVAC Phase 1 clinical evaluation. SO2 proved to be safe in guinea pigs and was more attenuated than BCG Pasteur in severe combined immunodeficiency (SCID) mice [16, 18]. In addition, SO2 conferred better protection than BCG Danish 1331 in a high-dose challenge long-term protection model in guinea pigs [16, 29]. SO2 also showed improved reduction of lung bacillary burden in rhesus macaques when compared to BCG [19]. Finally, immunogenicity data in mice showed that SO2 was able to induce a higher differentiation of antigen-specific $CD4^+$ T cells into central memory T cells, which correlated with longer protective efficacy in this model [37]. This latter result is especially important because some authors hypothesize that this inconsistent efficacy conferred by BCG may concern insufficient induction of long-lived memory T-cell responses [38].

5. Conclusions

Data shown provide evidence that MTBVAC is functionally and phenotypically comparable to its prototype SO2. The results of these studies fulfil the first and second Geneva consensus safety requirements for entry into clinical trials of live attenuated *M. tuberculosis* vaccines [20, 21]. The absence of front-line lipids, lack of ESAT-6 secretion and down-expression of the PhoP regulon, essential for virulence and pathogenesis of *M. tuberculosis*, may explain the satisfactory safety profile of MTBVAC.

Altogether, the improved protection levels against TB disease achieved by prototype SO2 and MTBVAC in mice, guinea pigs and non-human primates and the rigorous preclinical

safety and biodistribution data presented in this work have satisfied the Regulatory Authorities and enabled MTBVAC to be the first *M. tuberculosis* vaccine candidate to enter human clinical evaluation, positioning MTBVAC as a reliable vaccine for human use with potential to replace BCG.

Competing interests: AA, JG, BG and CM are co-inventors on a composition of matter patent “tuberculosis vaccine” filled by the University of Zaragoza. AP, CF and EP are employees of Biofabri, the exclusive licensee for MTBVAC. There are no other conflicts of interest.

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439

Figure legends

Figure 1. From SO2 to MTBVAC: step-by-step construction (A) and biochemical characterization of MTBVAC (B-D). (A) Deletions in *fadD26* and *phoP* genes were genetically engineered in SO2 strain. The final strain MTBVAC is a DIM-deficient *phoP* mutant which provides better assurance of genetic stability and has no antibiotic-resistance markers, fulfilling the Geneva consensus requirements. *phoP* and *fadD26* genes are represented as grey arrows, white rectangles illustrate antibiotic-resistance markers and black arrow-heads depicts *res* sites. Vertical discontinuous lines indicate the position of restriction sites used for strain construction and horizontal discontinuous lines depict DNA regions that are not to scale. **(B-C)** Thin-layer chromatography analysis showing that MTBVAC is devoid of cell-wall lipids DIM **(B)** and DAT/PAT **(C)**, as direct consequence of *fadD26* and *phoP* deletions, respectively. Compounds of interest are indicated by arrow-heads. **(D)** The absence of ESAT-6 secretion, characteristic of *M. tuberculosis phoP* mutants, was tested by Western blot.

Figure 2. Bridging efficacy studies of MTBVAC and SO2 in Guinea Pigs. Guinea pigs received a subcutaneous administration of the indicated doses of MTBVAC (white, grey and black diamonds) or SO2 (black circles), or not vaccinated (white squares), followed by an aerosol challenge with virulent H37Rv. At 4 weeks post-challenge, bacterial burden was assessed in lungs **(A)** and spleen **(B)**. Comparable CFU reduction was observed with SO2 and the three tested doses of MTBVAC. Data are expressed as Mean ± SEM and compared by non-parametric Mann-Whitney test. ***, $P < 0.001$.

Figure 3. MTBVAC safety in SCID mice. SCID mice received a single subcutaneous administration of MTBVAC (black), BCG Danish 1331 (dark grey) or BCG Pasteur (light grey) equivalent to 50 times the dose recommended for BCG in humans. A group inoculated with one single dose of virulent Mt103 was used as control (white). CFU in lungs **(A)** and spleen

(B) were determined at the endpoint of the experiment (13 weeks), except for Mt103-infected animals which died by week 6. All the animals inoculated with the vaccine strains survived until the end of the experiment and presented comparable bacterial burden. Data are expressed as Mean ± SEM and compared using unpaired Student's *t* test. ***, $P < 0.001$.

Figure 4. MTBVAC biodistribution profile. BALB/c mice were intradermally inoculated with MTBVAC (black diamonds) or BCG Danish 1331 (white circles) and bacterial load in lymph nodes was determined. A similar clearance profile was obtained for both strains. Data are expressed as Mean ± SEM and compared using unpaired Student's *t* test. *, $P < 0.05$; **, $P < 0.01$.

Figure 5. Protective efficacy of MTBVAC in mice. C57BL/6 mice received a subcutaneous administration of MTBVAC (black diamonds) or BCG Danish 1331 (white circles), or were not vaccinated (white squares), followed by an intranasal challenge with virulent H37Rv. At 4 weeks post-challenge, bacterial burden was assessed in lungs **(A)** and spleen **(B)**. Significant CFU reduction was observed in MTBVAC vaccinated animals compared to BCG. Data are expressed as Mean ± SEM and compared using unpaired Student's *t* test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Supplementary figure 1. Verification of *fadD26* deletion and subsequent elimination of the antibiotic-resistance marker. (A, C) Schematic representation of *fadD26* genomic region in Mt103 **(A, C)**, SO2Δ*fadD26*::*hyg* **(A)** and SO2Δ*fadD26* **(C)**. Location of specific primers is represented above by arrow-heads and approximated expected sizes for the PCR products are indicated below. **(B, D)** PCR analysis of the strains using the primer combinations indicated in left panels (A, C).

Supplementary figure 2. Verification of *phoP* deletion and subsequent elimination of the antibiotic-resistance marker. (A, C) Schematic representation of *phoP* genomic region

in Mt103 **(A, C)**, SO2Δ*fadD26*Δ*phoP*::*hyg* **(A)** and MTBVAC **(C)**. Location of specific primers is represented above by arrow-heads and approximated expected sizes for the PCR products are indicated below. **(B, D)** PCR analysis of the strains using the primer combinations indicated in left panels (A, C).

Supplementary figure 3. *In vitro* growth characterization of MTBVAC. (A) Growth kinetics of Mt103 (squares), SO2 (circles) and MTBVAC (diamonds) was studied in 7H9 liquid medium by measuring the optical density at 600 nm (OD_{600nm}) (filled symbols) and CFU counts (open symbols). **(B)** Colony morphology of the strains on solid 7H10 medium. No major differences in the growth rate of the three strains were observed in liquid broth, while colonies were smaller for both vaccine candidates on solid medium.

Supplementary figure 4. Bridging immunogenicity study of MTBVAC and SO2. BALB/c mice were inoculated with MTBVAC (black), SO2 (dark grey) or BCG Danish 1331 (light grey), or not vaccinated (white), and immunogenicity was measured as the percentage of $IFN\gamma^+$ $CD4^+$ splenocytes. MTBVAC and SO2 induce equivalent T-cell responses at all the studied post-inoculation time-points. Data are expressed as Mean ± SEM.

Supplementary methods

1. Construction of plasmids

E. coli strains, used for cloning processes, were grown at 37°C in LB broth or agar plates. Media were supplemented with ampicillin (100 μg/ml), gentamicin (Gm) (15 μg/ml), kanamycin (Km) (20 μg/ml) or hygromycin (Hyg) (50 μg/ml), when necessary. The same strategy was used for the construction of the plasmids for *fadD26* (pAZ5) and *phoP* (pAZ18) inactivation (table S1). DNA fragments containing the *fadD26* or *phoP* genes were amplified from *M. tuberculosis* H37Rv genomic DNA using primers fadD26F and fadD26R or phoPF and phoPR [12] (table S1), respectively, and cloned in pGEM-T Easy (Promega). An *EcoR*V-*BamH*I fragment from pWM27 [25], containing a *res*-Ω*hyg*-*res* cassette, was inserted between either *BamH*I and *EcoR*V sites in *fadD26* (1,511-bp deletion) or *EcoR*V and *Bcl*I in *phoP* (94-bp deletion). The fragments containing the deleted *fadD26* or *phoP* genes, and their flanking regions, were then released by digestion with *Xho*I and subsequently inserted in pJQ200-*xylE* [26] (table S1), a mycobacterial suicide plasmid containing the counter-selectable marker *sacB* and the reporter gene *xylE*.

A resolvase expression plasmid carrying a Km-resistance cassette, pAZ20 (table S1), was constructed by inserting the *BsaB*I-*Sph*I fragment from plasmid pPR23 [27] containing the *sacB* gene and a Gm-resistance cassette, between *Sph*I and *Sfo*I sites in pCG124 [25].

2 Construction of MTBVAC vaccine candidate

Except where indicated, mycobacterial strains were grown at 37°C in Middlebrook 7H9 broth (Difco) supplemented with ADC (Difco) and 0.05% Tween-80, or on solid Middlebrook 7H10 (Difco) supplemented with OADC (ADC plus oleic acid) (Difco). When required, Km, Hyg (20 μg/ml), Gm (10 μg/ml) or 2% (w/v) sucrose (Suc) were added to the media.

SO2 strain was electrotransformed with pAZ5 (table S1) and allelic exchange events were selected in two steps. Transformants were selected on Hyg-containing plates and single

colonies were assayed for Suc sensitivity. In the second step, two Hyg^R Suc^S colonies were propagated in liquid medium to allow a second recombination event to occur, and serial dilutions were plated on Suc-containing plates to select bacteria that had lost the *sacB* gene. To discard false-positives due to mutations in *sacB* gene, a XylE activity assay (by spreading a catechol solution) was performed. Hyg^R Suc^R clones were tested by PCR using various combinations of primers located either in the *fadD26* deleted region (fadD26I1, fadD26I2; table 1) or hybridizing in the *res* site (res1, res2; table S1) [25] and outside the *fadD26*-flanking regions cloned in pAZ5 (fadD26up, fadD26down; table S1 & Fig. S1A). One clone giving the pattern corresponding to allelic exchange was retained to continue the construction and named SO2Δ*fadD26*::*hyg* (Fig 1A & Fig. S1B). Then, to eliminate the Hyg-resistance marker, pWM19 [25] (table S1) was introduced in SO2Δ*fadD26*::*hyg* strain and transformants were selected at 30°C on Gm-containing plates. Two transformants were grown until saturation in liquid broth at 30°C to allow resolvase expression, and serial dilutions were plated on Suc-supplemented plates and incubated at 39°C forcing loss of pWM19. Several clones were tested for Hyg and Gm sensitivity and by PCR using primers fadD26F and fadD26R (table S1 & Fig S1C). Sequencing of the PCR product obtained from the selected clone SO2Δ*fadD26* confirmed the presence of one copy of *res* site as "scar" of the resolution process (Fig 1A & Fig. S1D).

Next step was the replacement of the Km-resistance cassette inserted in *phoP* gene in SO2 strain by a deletion containing a *res*-flanked cassette. SO2Δ*fadD26* was electrotransformed with pAZ18 (table S1) and allelic exchange events were selected as described above to obtain the strain SO2Δ*fadD26*Δ*phoP*::*hyg* (Fig. 1A) and PCR analysis was performed using various combinations of specific primers (res1, res2, phoPF, phoPR; table S1 & Fig S2A-B) to confirm homologous recombination. Finally, plasmid pAZ20 (table S1) was introduced in SO2Δ*fadD26*Δ*phoP*::*hyg* to generate the *phoP* unmarked mutant. Transformants were selected on Km-containing plates, grown until saturation in liquid medium and plated on Suc-supplemented plates. Clones obtained were tested by PCR using primers phoRF and phoPR

567 (table S1 & Fig. S2C) and one clone giving the appropriate PCR product was selected and
568 named MTBVAC (Fig. 1A & Fig. S2D).

569 **Table 1. Summary of the preclinical studies conducted with final MTBVAC product to**
570 **support entry into clinical trials**
571

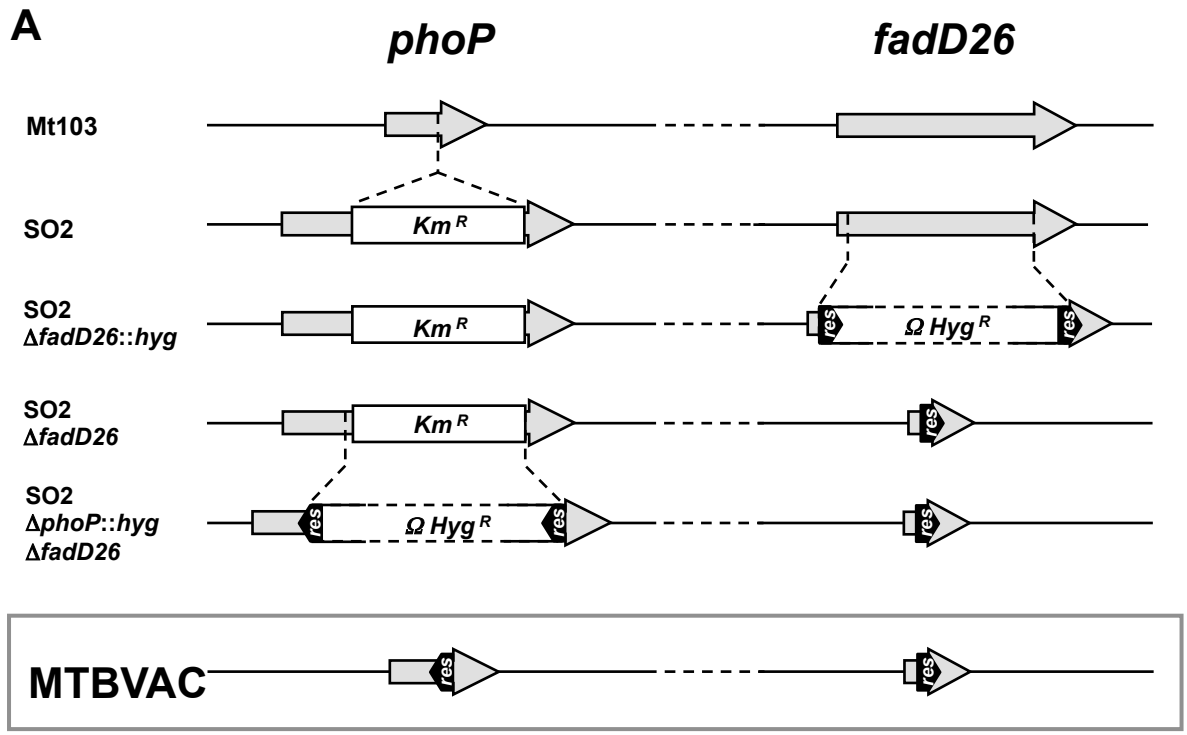
Study	Description	Animal species	Duration (weeks)	Laboratory	Results
Safety 50 human doses	Survival; CFU in lungs and spleen	SCID mice	13	Germans Trias I Pujol	Survival & CFU recovered equivalent to BCG
	Survival; histopathology	Guinea pigs	6	Biofabri	Survival & absence of TB lesions
Biodistribution, Excretion & Shedding	CFU in lungs, liver, kidneys, spleen, local lymph nodes, brain and gonads	BALB/c mice	22-24	University of Zaragoza	Biodistribution equivalent to BCG
	Excretion in urine and stool				Absence of excretion
	Excretion in urine and stool & Shedding at vaccination site	Guinea pigs	7	PHE	Absence of excretion
Protection	CFU in lungs and spleen	C57BL/6 mice	4 post-vaccination	University of Zaragoza	Protection superior to BCG

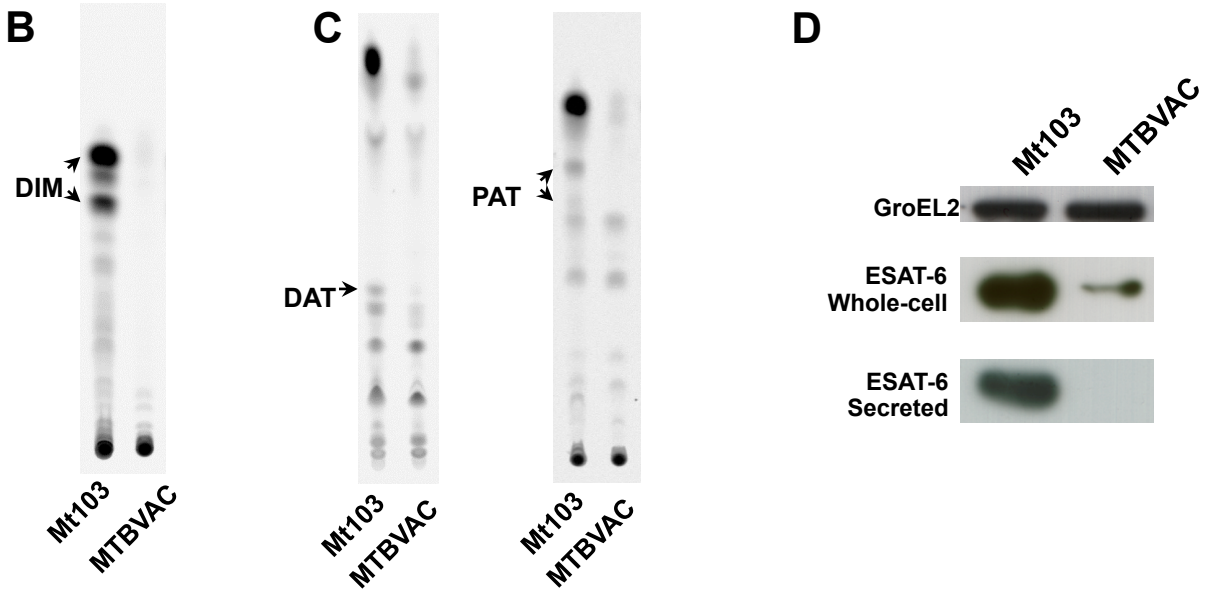
572

573 **Table S1. Bacterial strains, plasmids and primers used in this study**

Mycobacterial strains	**Description**	**Reference**
Mt103	Parental *M. tuberculosis* clinical isolate	[24]
SO2	*phoP* insertion mutant in Mt103 (Km^R)	[11]
MTBVAC	Double unmarked *phoP* and *fadD26* deletion mutant in Mt103 (constructed from SO2)	This work
BCG Pasteur	Laboratory reference BCG strain	Our laboratory collection
BCG Danish 1331	Only licensed BCG vaccine in Europe	Our laboratory collection
Plasmids	**Description**	**Reference**
pJQ200-*xylE*	Mycobacterial suicide plasmid carrying the counterselectable marker *sacB* and the reporter gene *xylE* (gm^R)	[26]
pWM27	Plasmid harboring the *res*-Ωhyg^R-*res* cassette (hyg^R)	[25]
pAZ5	pJQ200-*xylE* derivative containing a deleted copy of *M. tuberculosis fadD26* gene (hyg^R, gm^R)	This work
pAZ18	pJQ200-*xylE* derivative containing a deleted copy of *M. tuberculosis phoP* gene (hyg^R, gm^R)	This work
pWM19	Mycobacterial thermo-sensitive plasmid for resolvase γδ expression, harboring the counterselectable marker *sacB* (hyg^R, gm^R)	[25]
pCG124	Mycobacterial plasmid for resolvase γδ expression (km^R)	[25]
pPR23	Mycobacterial thermo-sensitive plasmid carrying the counterselectable marker *sacB* (hyg^R, gm^R)	[27]
pAZ20	Mycobacterial plasmid for resolvase γδ expression, harboring the counter selectable marker *sacB* (km^R, gm^R)	This work
Primers	**Nucleotide sequence**	**Reference**
fadD26F	5'-CTCGAGTTCTCTATCCGTGTATTC-3'	This work
fadD26R	5'-CTCGAGGTTGGTCTTGACAG-3'	This work
phoPF	5'-AATCTAGATCAAGCATCAGCCGAGGTAC-3'	[12]
phoPR	5'-AATCTAGAGATCACCCGAACGTAGAACC-3'	[12]
fadD26I1	5'-CACGAATGTCATTGCCAATG-3'	This work
fadD26I2	5'-GCTTGAGCATGACCTCTTCG-3'	This work
fadD26up	5'-CAACGCAAGACGACATGG-3'	This work
fadD26down	5'-GCACCGTCTTGATGAAGC-3'	This work
res1	5'-CTAGAGCAACCGTCCGAAATATTATAA-3'	[25]
res2	5'-GATCTCATAAAAATGTATCCTAAATCAAATATC-3'	[25]
phoRF	5'- AATCTAGAGGGCAAGGGCAACAAGGAAC-3'	This work

574


A
phoP
fadD26
Mt103
SO2
Km R
SO2
ΔfadD26::hyg
Km R
Ω Hyg R
res
SO2
ΔfadD26
Km R
res
SO2
ΔphoP::hyg
ΔfadD26
res
Ω Hyg R
res
res
MTBVAC
res
res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DIM
Mt103
MTBVAC
C
DAT
PAT
Mt103
MTBVAC
Mt103
MTBVAC
D
Mt103
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GroEL2
ESAT-6
Whole-cell
ESAT-6
Secreted

Figure 1

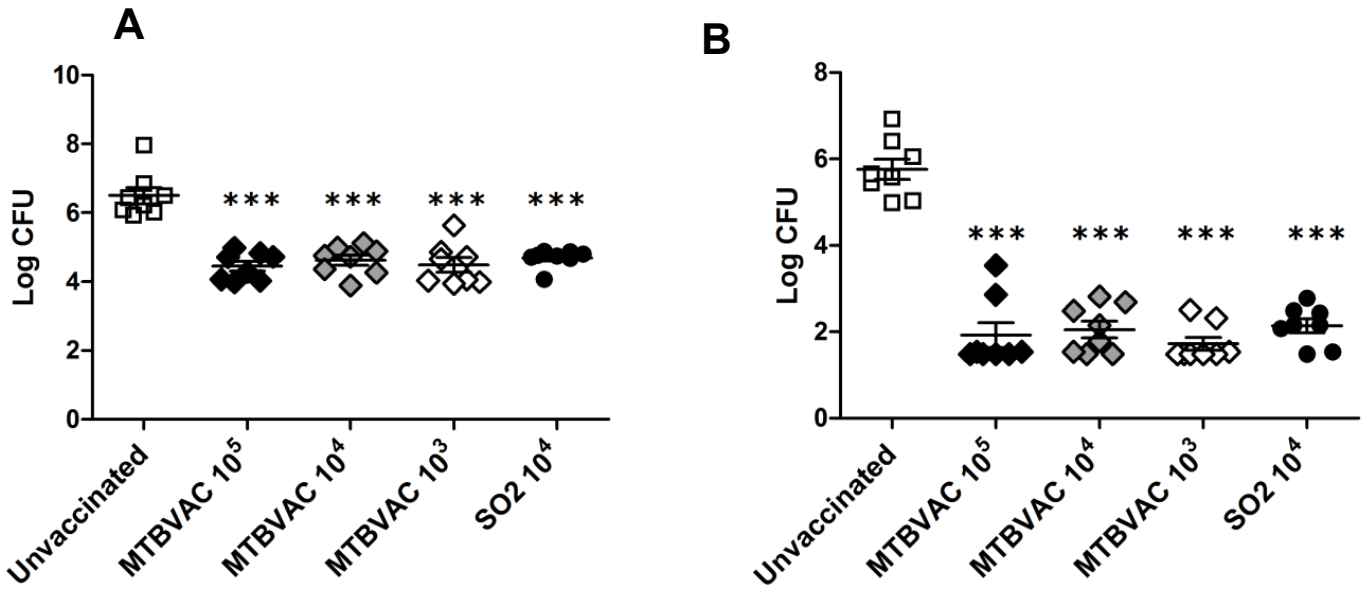

A
B
Log CFU
***
Unvaccinated
MTBVAC 10^5
MTBVAC 10^4
MTBVAC 10^3
SO2 10^4

Figure 2

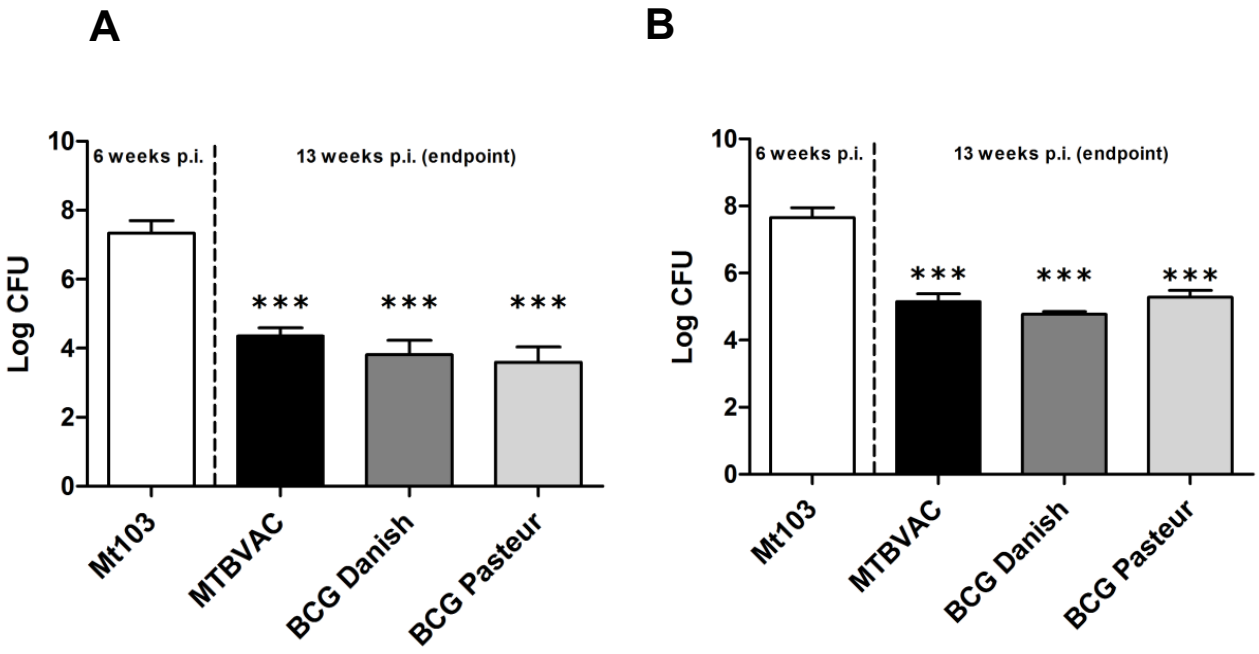

A
B
6 weeks p.i.
13 weeks p.i. (endpoint)
Log CFU
10
8
6
4
2
0
***
Mt103
MTBVAC
BCG Danish
BCG Pasteur

Figure 3

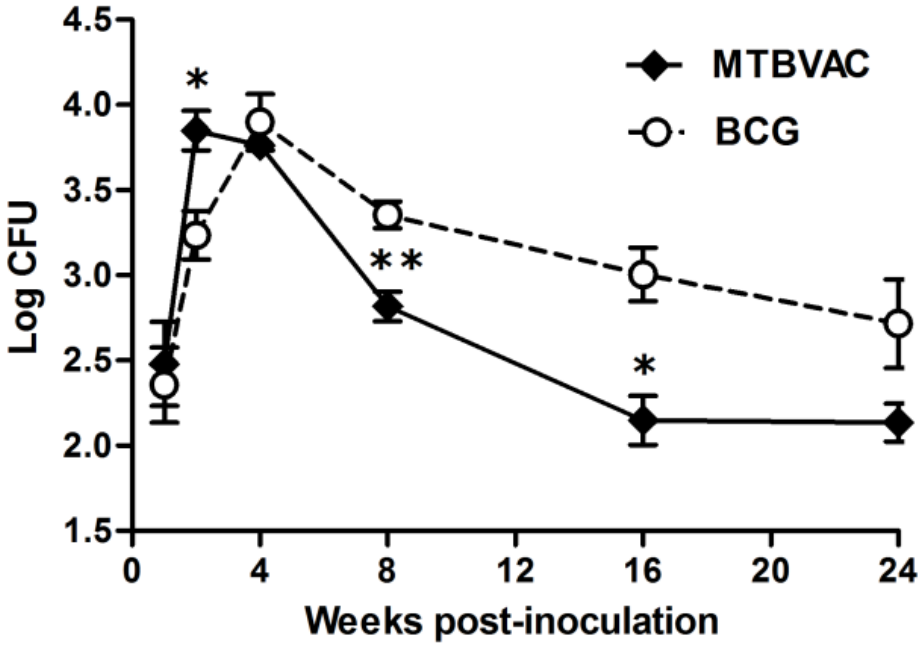

4.5
4.0
3.5
3.0
2.5
2.0
1.5
Log CFU
0
4
8
12
16
20
24
Weeks post-inoculation
MTBVAC
BCG
*
**
*

Figure 4

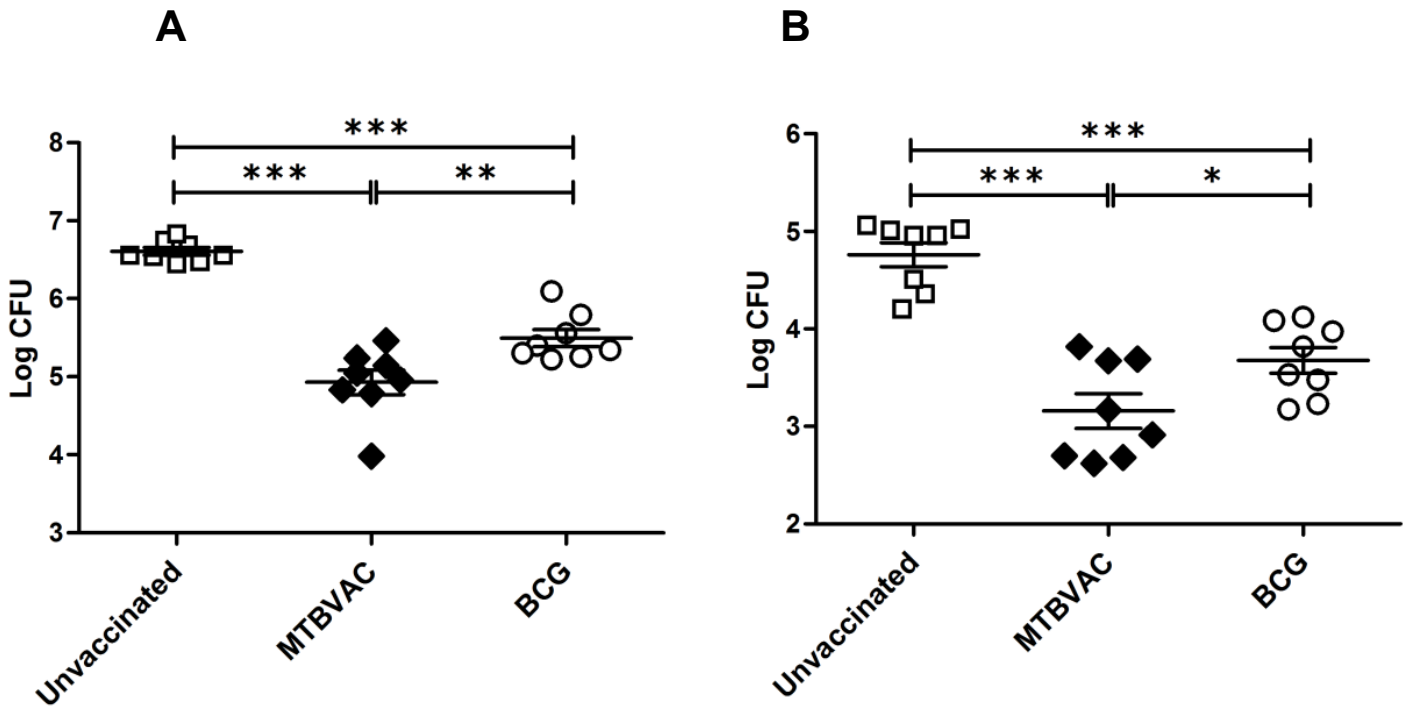


Figure 5

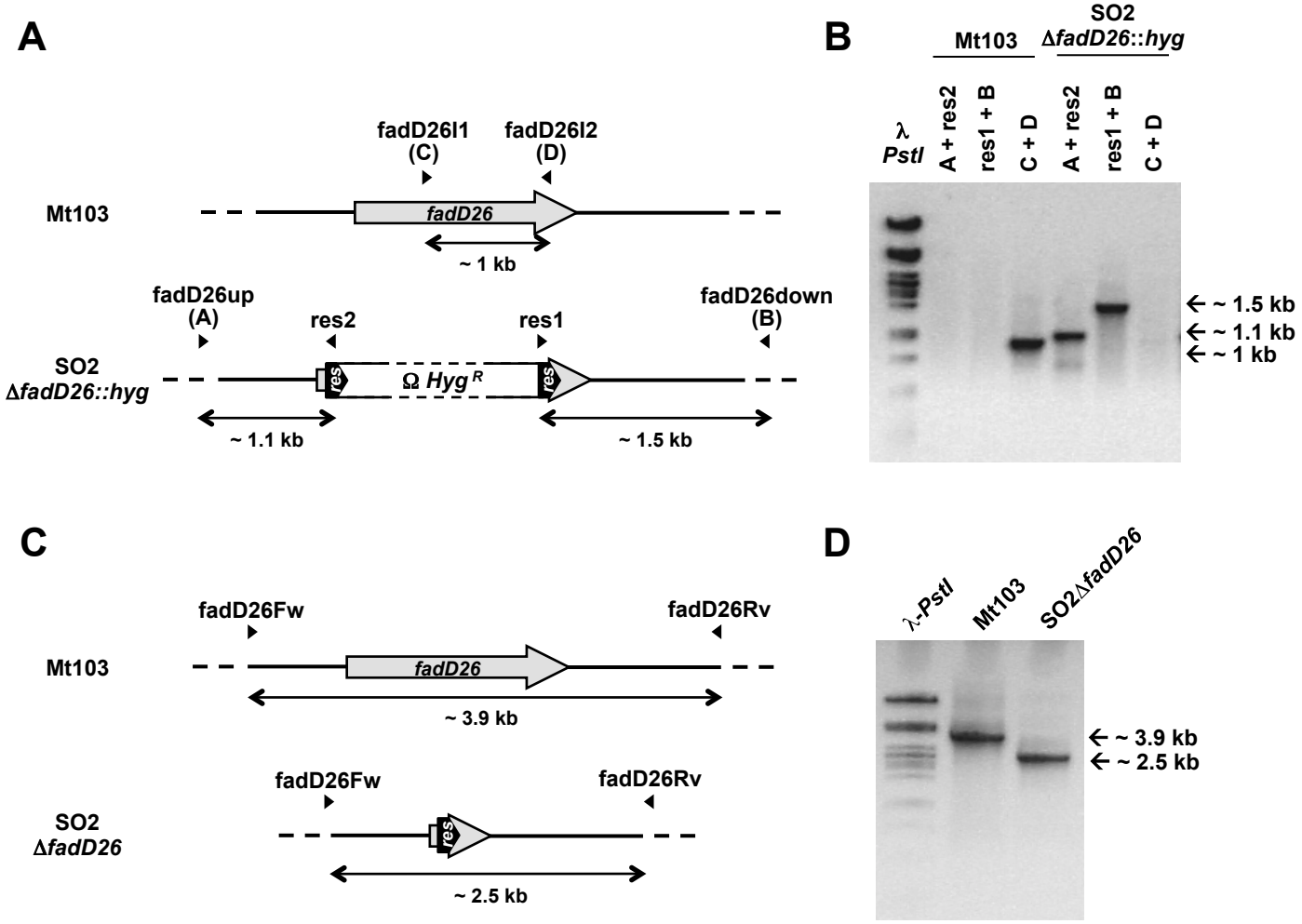

A
fadD26I1
(C)
fadD26I2
(D)
Mt103
fadD26
~ 1 kb
fadD26up
(A)
res2
res1
fadD26down
(B)
SO2
ΔfadD26::hyg
res
Ω Hyg R
res
~ 1.1 kb
~ 1.5 kb
B
Mt103
SO2
ΔfadD26::hyg
λ
PstI
A + res2
res1 + B
C + D
A + res2
res1 + B
C + D
~ 1.5 kb
~ 1.1 kb
~ 1 kb
C
fadD26Fw
fadD26Rv
Mt103
fadD26
~ 3.9 kb
fadD26Fw
fadD26Rv
SO2
ΔfadD26
res
~ 2.5 kb
D
λ-PstI
Mt103
SO2ΔfadD26
~ 3.9 kb
~ 2.5 kb

Figure S1

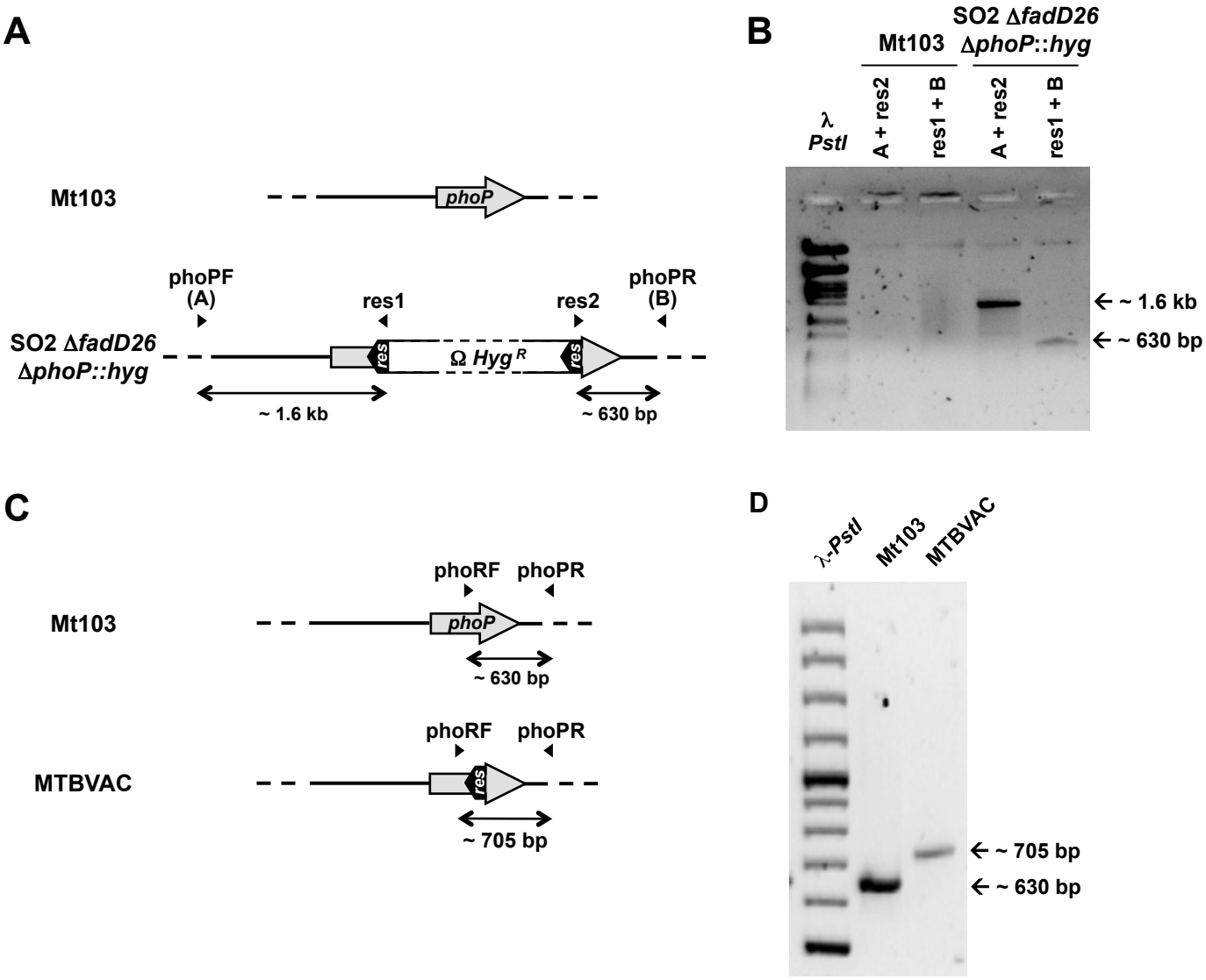
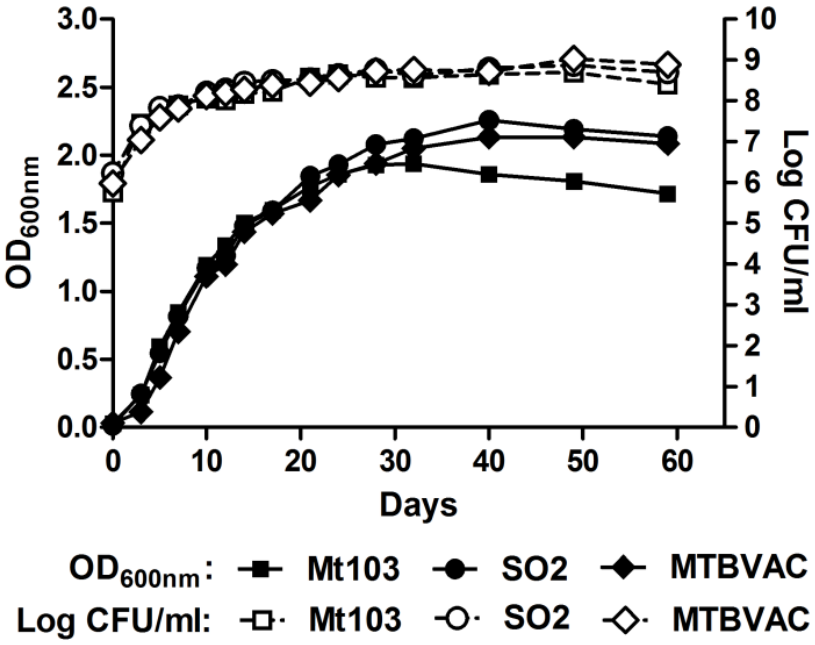

A
Mt103
phoP
phoPF
(A)
res1
res2
phoPR
(B)
SO2 ΔfadD26
ΔphoP::hyg
res
Ω Hyg R
res
~ 1.6 kb
~ 630 bp
B
SO2 ΔfadD26
ΔphoP::hyg
Mt103
λ
PstI
A + res2
res1 + B
A + res2
res1 + B
~ 1.6 kb
~ 630 bp
C
phoRF
phoPR
Mt103
phoP
~ 630 bp
phoRF
phoPR
MTBVAC
res
~ 705 bp
D
λ-PstI
Mt103
MTBVAC
~ 705 bp
~ 630 bp

Figure S2

A


3.0
2.5
2.0
1.5
1.0
0.5
0.0
OD600nm
10
9
8
7
6
5
4
3
2
1
0
Log CFU/ml
0
10
20
30
40
50
60
Days
OD600nm: Mt103 SO2 MTBVAC
Log CFU/ml: Mt103 SO2 MTBVAC

B

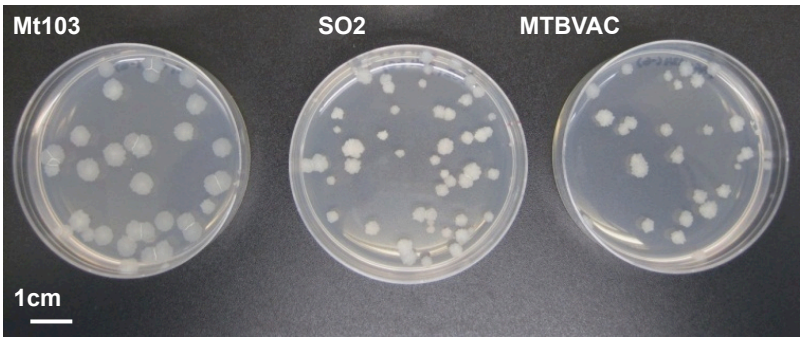

Mt103
SO2
MTBVAC
1cm

Figure S3

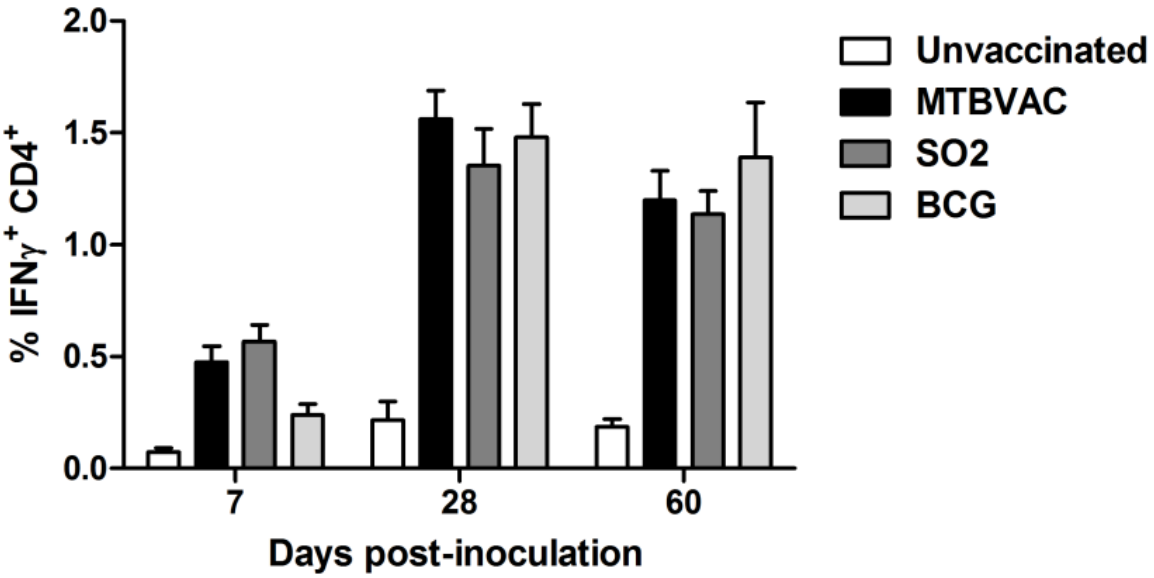


Figure S4

Arbues *et al*